

# Material-based regulation of the myofibroblast phenotype

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## Abstract

Fibroblast growth factor receptor (FGFR) activation by basic fibroblast growth factor (FGF-2) serves to naturally repress the myofibroblast activation of valvular interstitial cells (VICs). Co-receptors for FGF-2, the heparan sulfate proteoglycans (HSPGs), are key participants in the formation of active FGF-2 signaling complexes. Bioactive environments regulating the myofibroblast phenotype were created by utilizing heparin glycosaminoglycan as a competitive inhibitor of HSPGs. First, soluble heparin was delivered to compete with cell-surface HSPG for the binding of FGF-2. Exogenous soluble heparin prevented serum-dependent activation of the classic mitogen-activated protein kinase (MAPK) and induced myofibroblast alpha smooth muscle actin ( $\alpha$ SMA) expression and collagen production. Next, heparin-functionalized hydrogel cell substrates were polymerized from vinyl-modified precursors and rendered adhesive through incorporation of RGDS peptide. Culture of VICs on heparin-modified gels induced  $\alpha$ SMA expression and inhibited MAPK activity compared to control gel substrates lacking heparin. Additionally, heparin-functionalized gels continued to induce  $\alpha$ SMA expression in serum-free culture conditions, suggesting that bioactivity was independent of exogenous soluble mediators. Biomaterial scaffolds targeting cell surface growth factor receptors are a promising new direction for regulating cell functions in tissue-engineering applications.

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## 1. Introduction

The successful regeneration of functional tissue equivalents depends on the development of instructive cellular microenvironments that can influence the evolution of cellular phenotypes and production of extracellular matrix (ECM). In particular, it is critical to understand the extracellular signals that link to intracellular events and regulation of gene expression. For example, valvular interstitial cell (VIC) phenotype is dynamically converted from a quiescent state into an activated smooth muscle cell phenocopy, known as the myofibroblast, under the direction of signals derived from the ECM and associated growth factors. Myofibroblasts, historically identified by their contraction of granulation tissue [1], are intimately involved in development, wound repair, and ECM remodeling. Understanding the molecular pathways implicated in

the process of myofibroblast activation is a critical first step in developing matrix niches that can regulate the myofibroblastic phenotype, and recognizing factors that *inhibit* or *reverse* this process are equally important as prolonged presence of myofibroblasts can contribute to the formation of fibrotic tissue [2]. To harness the unique synthetic and secretory properties of the myofibroblast for tissue production in regenerative applications, we sought to develop an extracellular culture platform based on functionalized hydrogels to temporally regulate the VIC myofibroblast phenotype.

Controlled delivery of growth factors that induce myofibroblast activation has been the primary approach to accelerate cell-based aortic valve regeneration. Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), in particular, is a potent physiologic stimulus for induction of smooth muscle cell gene expression within myofibroblast precursor cells [3]. Less understood are the factors that inhibit myofibroblast activation and maintain the VIC population in a quiescent state. By focusing on factors mediating myofibroblast

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deactivation, we hypothesized that a new axis of regulation could be created to either suppress or promote the myofibroblast phenotype.

Basic fibroblast growth factor (bFGF, also known as FGF-2) appears to modulate wound-healing responses by inhibiting persistent myofibroblast activation in several connective tissues [4–6], including recent work linking its inhibition of myofibroblast activation in the aortic valve [7]. FGF-2 signals through a dual receptor system comprised of high affinity cell-surface transmembrane receptor tyrosine kinases (RTKs/fibroblast growth factor receptor (FGFRs)) and lower affinity heparan sulfate proteoglycan (HSPG) receptors, such as the glypican and syndecan HSPG families that are expressed with tissue and context specificity [8]. When the heparan sulfate chains of HSPGs are chemically desulfated, exogenous heparin can act as a substitute and restore growth factor binding [9]. A multi-molecular complex of HSPG, FGF, and the FGFR is assembled to activate the RTK functionality of the FGFR cytoplasmic tail [9,10]; however, the full activity of FGF-2 is mediated not only by receptor interactions but also by internalization of ligand-receptor complexes [11–13]. Among activation of other signaling cascades, FGF-2 leads to the downstream activation of the “classical” mitogen-activated protein kinase (MAPK) family consisting of extracellular signal-related kinases 1 and 2 (ERK1/2) [14]. As apposed to delivering either TGF- $\beta$ 1 or FGF-2 to regulate the myofibroblast phenotype, we propose that myofibroblast activation can be controlled by manipulating the fidelity of the MAPK pathway, and that the FGF receptors themselves can be targeted with material interactions to mediate changes in MAPK pathway activity.

As FGF-2 is one of few factors identified to regulate myofibroblast deactivation, a material that could control the local environment around the FGFR would enable manipulation of downstream signaling pathways and temporal regulation of myofibroblast activity. Given the critical role of cell-surface heparan sulfates in FGF signaling and internalization, we hypothesized that exogenous heparin could be used to directly manipulate the responsiveness of the FGF receptors. To our knowledge, this represents the first example proposing to regulate growth factor receptor activity in a manner independent of the FGF protein ligand. Two scenarios were explored; in the first soluble heparin was delivered in monolayer culture to compete with cell-surface HSPG, and in the second heparin was covalently immobilized within hydrogel microenvironments to interact with cell-surface FGFRs. These approaches are diagrammed in Fig. 1.

To test the link between heparin and myofibroblast activation, the patency of FGFR signal transduction through MAPK/ERK was characterized in the presence and absence of high-dose exogenous heparin. Exogenous soluble heparin was found to disrupt phosphorylation of ERK and lead to an activated myofibroblast VIC phenotype as characterized by the expression of alpha smooth muscle actin ( $\alpha$ SMA). Additionally, exogenous FGF-2 delivery was found to rescue the defect in ERK phosphorylation created by soluble heparin and prevent myofibroblast activation, suggesting a causal link between MAPK inhibition and myofibroblast activation. Next, heparin was covalently incorporated into poly(ethylene glycol) (PEG)-based hydrogels, and these heparin-modified PEG gels were rendered cell adhesive via addition of the integrin-ligand RGDS peptide. VICs cultured on gels

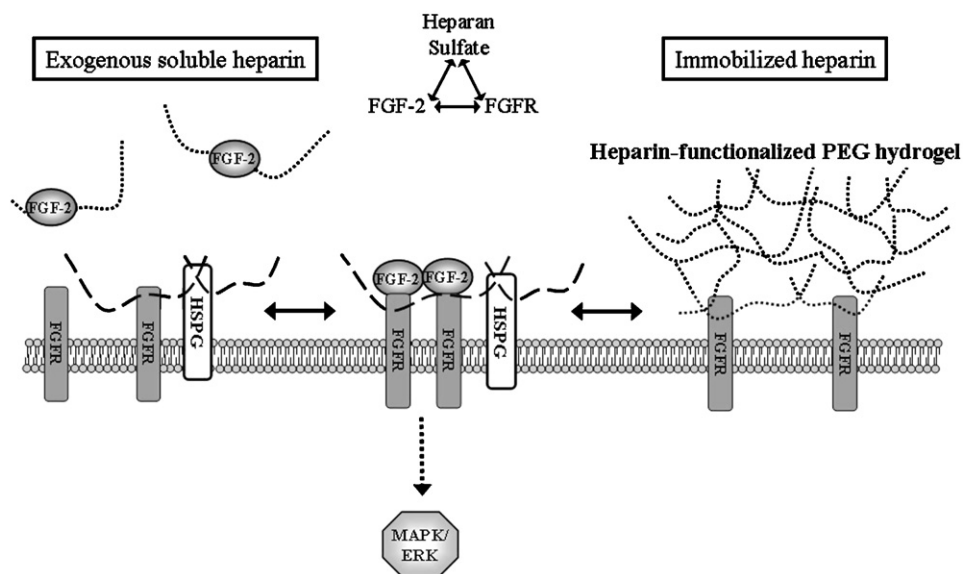


Fig. 1. Basic fibroblast growth factor (FGF-2) acts by binding both cell-surface heparan sulfate proteoglycans (HSPGs, ---) and FGF receptors (FGFRs), and activating downstream signaling molecules, such as mitogen activated protein kinase (MAPK)/extracellular-signal regulated kinase (ERK). Exogenous heparin (----), either supplied as a soluble competitor (left) or immobilized within a material (right), can be used to manipulate FGF-2 signaling.

containing immobilized heparin underwent spontaneous myofibroblast activation but remained responsive to FGF-2 deactivation stimulus, suggesting that heparin-modified gels may have utility as biomaterial niches for manipulation of the myofibroblast phenotype by fundamentally lowering the threshold for myofibroblast activation through material/growth factor receptor interactions.

## 2. Methods

### 2.1. Materials

FGF-2 and heparin (MW 18 kD, isolated from porcine intestinal mucosa) were purchased from Sigma. Mouse monoclonal anti- $\alpha$ SMA, clone 1A4 (ab7817) was purchased from Abcam Inc., and used at 1:75 dilution. Alexa Fluor<sup>®</sup> 488 F(ab')<sub>2</sub> goat anti-mouse IgG (GAM-488), goat anti-mouse horseradish peroxidase conjugate (GAM-HRP), and goat anti-rabbit HRP (GAR-HRP) were purchased from Molecular Probes and used at 1:400 dilution. Anti-ACTIVE<sup>®</sup> MAPK antibody, specific for the dual phosphorylated forms of p44/ERK1 and p42/ERK2, was purchased from Promega. Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich.

### 2.2. Cell culture

Aortic valve leaflets were surgically isolated from porcine hearts purchased from Quality Pork Processors Inc. (Austin, MN), and VICs were isolated by sequential collagenase digestion as previously described [15]. VICs were cultured in growth media consisting of 15% fetal bovine serum (FBS), 2% penicillin/streptomycin, 0.2% gentamicin in Media 199 at 37 °C in a 5% CO<sub>2</sub> environment.

### 2.3. VIC seeding

VICs were isolated as stated above, cultured at subconfluent densities, and used at passage 1–2. Seeding was performed in black tissue-culture treated 96-well flat-bottom plates at 50,000 cells/cm<sup>2</sup> for phospho-ERK assays, at 20,000 cells/cm<sup>2</sup> for  $\alpha$ SMA expression assays, and at 25–30,000 cells/cm<sup>2</sup> on hydrogel surfaces. For cell attachment, 12 h were allowed before replacing growth media with conditioned media containing exogenous factors, for example media containing heparin (0–1000  $\mu$ g/ml), FGF-2 (0–100 ng/ml), chlorate (0–25 mmol/L), hyaluronic acid (HA, from *Streptococcus equi*) or chondroitin sulfate (ChS) (0–200  $\mu$ g/ml). Conditioned media was incubated with cell cultures for 48 h before final analysis, except for phospho-ERK assay which was incubated for only 30 min to examine short-term phosphorylation events and prevent culture over-confluence and nodule formation, which can occur at high seeding densities.

### 2.4. Matrix production

The effect of FGF-2 (1–100 ng/ml) on VIC collagen matrix production was measured by <sup>3</sup>H-proline incorporation over a 96 h period with heparin treatment (100  $\mu$ g/ml). For assay, treated cells were removed from culture, extensively washed, and cell-associated proline was removed with a cell lysis step in 25 mM NH<sub>4</sub>OH. Remaining ECM was then digested for 4 h with papain protease at 37 °C. ECM digests were combined with scintillation fluid, and radioactivity determined by scintigraphy (Beckman LS 6500, Beckman Instruments Inc.). DNA from corresponding samples was quantified using the PicoGreen<sup>®</sup> dsDNA (Invitrogen Corp.) assay after cell lysis in GloLysis Buffer (Promega Corp.). Data is presented as the counts per minute (cpm) normalized to DNA content of each sample to account for changes in proliferation.

### 2.5. Activation of FGF-2 intracellular phospho-relay system: ERK phosphorylation

The CASE<sup>™</sup> Kit for ERK1/2 (SuperArray Bioscience Corp.) was used to monitor changes in phosphorylation of ERK1 and ERK2 in cultured VICs treated with FGF-2 (0–10 ng/ml) in the absence or presence of 100  $\mu$ g/ml heparin for a 30 min period. Significant modifications made to the manufacturer's instructions are outlined below. For each condition, 12 identical wells were seeded with VICs. Six of these wells were labeled with rabbit anti-phospho-ERK1/2 coupled with GAR-HRP, the remaining six wells were labeled with rabbit anti-total-ERK1/2 coupled with GAR-HRP. The fluorogenic HRP substrate, QuantaBlu<sup>™</sup> (Pierce), was used to quantify the signal from phospho-ERK and total-ERK on an automated plate reader (PerkinElmer Wallac Victor<sup>2</sup> Counter), and these were normalized on a per well basis to DNA content, as quantified by PicoGreen<sup>®</sup> dsDNA assay on samples after papain digestion. Results for phosphorylation of ERK are presented as follows:

$$\% \text{ ERK activation} = \frac{\text{Phospho ERK/DNA content}}{\text{Total ERK/DNA content}}.$$

### 2.6. Inhibition of HSPG sulfation

The activity of sulfate adenyltransferase was inhibited and the sulfate donor 3'-phosphoadenosyl-5'-phosphosulfate was depleted using sodium chlorate treatment (NaClO<sub>3</sub>), preventing sulfate donation to newly synthesized GAG chains [16]. Subconfluent VIC cultures were treated with NaClO<sub>3</sub> (0–25 mM) in sulfate-free/serum-free DMEM (GIBCO-BRL) for 72 h, and then again with sulfate-free/serum-free DMEM with and without 10 ng/ml FGF-2 for 48 h before assay of  $\alpha$ SMA protein as stated above.

### 2.7. Synthesis of methacrylated heparin (Hep-MA), poly(ethylene glycol) diacrylate (PEG-DA), and CRGDSG peptide

Heparin was modified with methacrylated groups following previously reported protocols [17,18]. Briefly, 10% (w/v) heparin in dH<sub>2</sub>O was combined with methacrylic anhydride at 40-fold molar excess. Using 4 N NaOH, the pH of the reaction mixture was adjusted to 7.5 and stirred vigorously at room temperature for 24 h. Methacrylated heparin was precipitated with cold 95% ethanol, filtered, dried, dialyzed (Spectrum, MWCO 1000) for 24 h against dH<sub>2</sub>O to remove residual methacrylic anhydride, and lyophilized. <sup>1</sup>H NMR analysis revealed an average of 26% methacrylation per disaccharide repeat unit. PEG-DA was synthesized as previously described [19], and <sup>1</sup>H NMR analysis revealed 87% acrylation. The product was dialysed against distilled water (MW cutoff 1000 Da), frozen, and lyophilized. The peptide CRGDSG (N-terminus to C-terminus) was synthesized using solid-phase methods (Applied Biosystems Peptide Synthesizer, model 433A), UV-monitoring, and HBTU (2-(1H-benzotriazol-1,1,3,3-tetramethyluroniumhexafluorophosphate) activation coupling. The peptide was cleaved from the resin support and deprotected using a cocktail of 5% phenol, 5% water, and 2.5% triisopropylsilane in trifluoroacetic acid, washed and precipitated in cold ethyl ether, redissolved in distilled water, and lyophilized.

### 2.8. Hydrogel adhesion studies

Polymer solutions consisting of 10 wt% PEG-DA monomer, 1.5 mm of CRGDSG peptide sequence, 0.5 mm of methacrylated GAG (heparin or ChS), and 0.05 wt% photoinitiator (Irgacure<sup>®</sup> 2959) in phosphate-buffered saline (PBS) were sterilized by 0.2- $\mu$ m syringe filtration and polymerized between two sterile glass slides under 365 nm ultraviolet light for 10 min to form a hydrogel sheet. Hydrogel disks were fabricated by using 7 mm biopsy punch (diameter = 7 mm, thickness = 1 mm). In contrast to previous work with RGD-functionalized gels made from

acrylated RGDS [20], the CRGDSSG peptide was incorporated into the network via mixed-mode reaction of the thiol containing cysteine residue and divinyl PEG [21]. The hydrogel disks were placed in a 24-well tissue-culture treated polystyrene plate and swelled in VIC growth media overnight. The disks were washed with PBS the next day, and VICs were seeded on the surfaces of the disks at 25,000–30,000 cell/cm<sup>2</sup>. Cells were incubated for 48 h at 37 °C before fixation in formalin and staining for  $\alpha$ SMA or phospho-ERK.

### 2.9. Cell lysis and western blotting

VICs from hydrogel adhesion studies were trypsinized off gels, centrifuged at 1000 rpm to obtain a cell pellet, lysed in Triton-X 100 lysis buffer (1% Triton X-100, 20 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 150 mM sodium chloride, 1 mM magnesium chloride, 1 mM calcium chloride and 10  $\mu$ g/ml Protease Inhibitor Cocktail Set III (EMD Biosciences)) and centrifuged at 16,000g for 10 min at 4 °C. Supernatants were flash frozen and stored at –80C. Analysis of protein content was performed with the Coomassie Plus Assay (Pierce) and BSA standard, and equal protein quantities were separated by gel electrophoresis (12% Tris-HEPES-SDS Precise™ Gels, Pierce) and electroblotted onto PVDF membranes (Bio-Rad). Precision Plus Protein™ Kaleidoscope standards (Bio-Rad) were used to verify molecular weights. GAM-HRP and Opti-4CN Detection Kit (Bio-Rad) were used to detect  $\alpha$ SMA. ImageJ densitometry software (Version 1.6, NIH) was used to obtain

semi-quantitative values for expression levels based on their relative intensities [22]. Expression intensity values were normalized to the control and are presented as fold change from control.

### 2.10. Statistics

Data is presented as mean  $\pm$  standard deviation. At a minimum, four samples were averaged for each data point. Data was compared using a two-tailed, unpaired *t*-test, and *p* values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Soluble heparin dose determines VIC $\alpha$ SMA expression

Heparan sulfates located on cell-surface proteoglycans are crucial participants in the formation of signaling FGF/FGFR multi-macromolecular complexes [9]. Heparin, a sulfated glycosaminoglycan (GAG) isolated from mast cells, can also participate in the formation of these complexes when applied exogenously. Because soluble heparin can freely diffuse, its concentration can determine the extent of FGF-2 binding to FGFRs [23]. In Fig. 2A, we

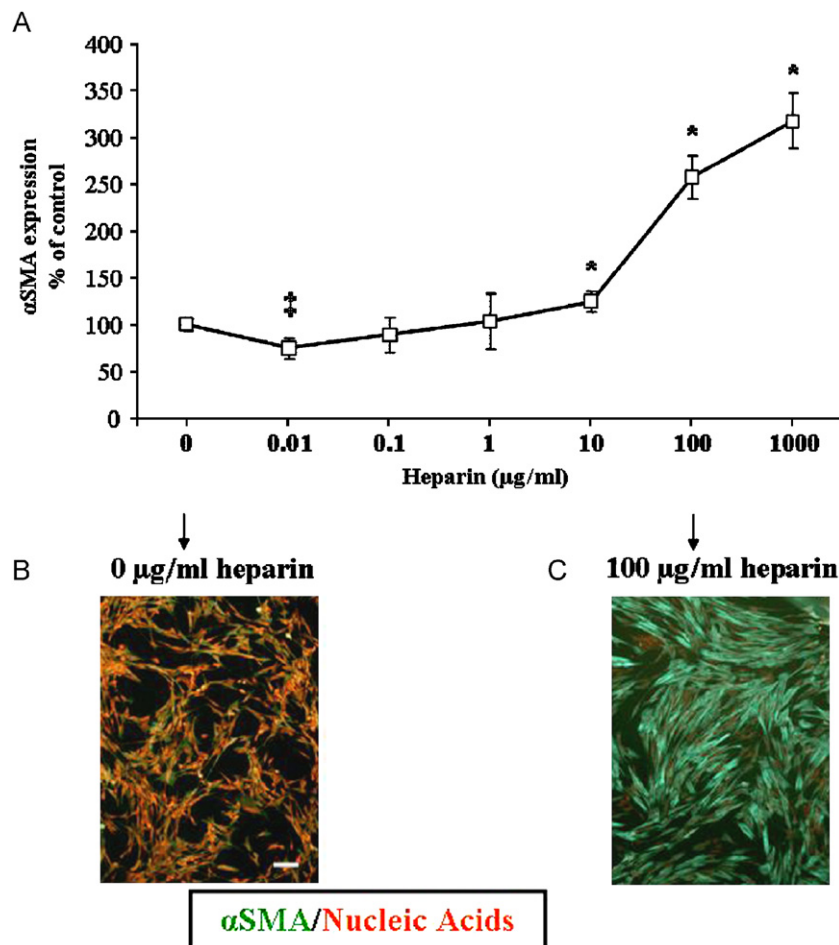


Fig. 2. (A) Valvular interstitial cell (VIC)  $\alpha$ SMA expression as a function of soluble heparin concentration (0–1000  $\mu$ g/ml). Above 10  $\mu$ g/ml,  $\alpha$ SMA expression is significantly elevated compared to levels of untreated controls; however  $\alpha$ SMA expression is depressed at 0.01  $\mu$ g/ml. (\*Indicates values significantly higher than control, <sup>‡</sup>indicates values significantly lower than control, *p* < 0.05.) Immunofluorescent micrographs labeling  $\alpha$ SMA (green) and nucleic acids (red) of control (B) and heparin-treated cells (C) (100  $\mu$ g/ml). Scale bar 10  $\mu$ m.

explore the concentration-dependent effects of soluble heparin on VIC myofibroblast activation.

Myofibroblasts are unique cells that have acquired ultrastructural features normally characteristic of smooth muscle cells, such as cytoskeletal stress fibers containing  $\alpha$ SMA, which serves as a marker of the activated myofibroblast. Congruent with previous results demonstrating that the appropriate dose of heparin can enhance FGF activity [24], the lowest concentration of heparin tested (0.01  $\mu$ g/ml) resulted in significantly decreased  $\alpha$ SMA levels. Above 10  $\mu$ g/ml, however, heparin treatment produced elevated  $\alpha$ SMA expression ( $p < 0.05$ ). This unique dose–response correlates with previously published data illustrating either potentiation or inhibition of FGF-2 signaling by soluble heparin depending on dose [24]. At low concentrations, heparin associates with the cell surface and can increase the formation of FGF/FGFR signaling complexes. However, increasing the concentration of heparin into the micromolar range reduces the binding of FGF to its receptor [23,24].

To discern if myofibroblast activation seen in response to heparin was shared with other acidic GAGs, exogenous ChS and HA were delivered to VICs at concentrations ranging from 0–200  $\mu$ g/mL. In results not shown, neither ChS nor HA affected VIC expression of  $\alpha$ SMA. As neither ChS nor HA bind FGF-2 or its receptor, these results confirmed our rationale to use heparin as a HSPG mimic.

### 3.2. Excess soluble heparin disrupts FGF-2 signal transduction through MEK/ERK

ERK1 and ERK2 (also known as p44/p42 MAPKs) are activated by dual phosphorylation of threonine and tyrosine sites by the MAPK kinases MEK1 and MEK2 after FGF-2 signaling [25]. To examine the effects of exogenous heparin on the activation of the MAPK pathway, the dual phosphorylation status of ERK was monitored while stimulating VICs with FGF-2 in the absence and presence of high-dose exogenous soluble heparin (100  $\mu$ g/ml). This concentration of heparin was chosen based on results in Fig. 2A indicating that 100  $\mu$ g/ml induced a  $\approx 2.5$ -fold induction in  $\alpha$ SMA expression, and based on a molecular weight of 17 kDa, this concentration corresponds to approximately 6  $\mu$ mol/L heparin.

In Fig. 3, the phosphorylation of ERK is presented as a function of FGF-2 concentration (open squares). FGF-2 has a molecular weight of 16.4 kDa [26], and the concentrations of 0.1–10 ng/ml used in this study correspond to a molar concentration range of 6–600 pmol/L. Fig. 3 demonstrates that ERK dual-phosphorylation increases in a dose-dependent fashion with increasing FGF-2. In this study, FGF-2 was added to serum-containing media. As such, the results presented here represent MAPK activation by both serum mitogens and by exogenously supplied FGF-2.

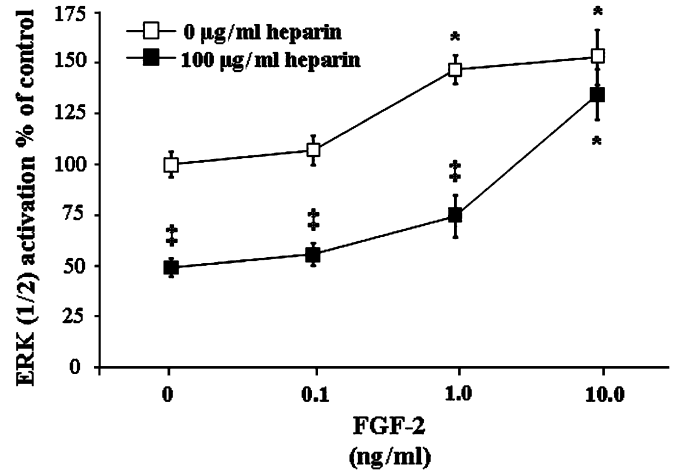


Fig. 3. Activation of ERK1 and ERK 2 mitogen-activated protein kinases. Dual phosphorylation of ERK1/2 reported as a function of FGF-2 concentration (0–10 ng/ml) in the presence ( $\square$ ) and absence ( $\blacklozenge$ ) of soluble heparin (100  $\mu$ g/ml). (\*Indicates values significantly higher than control,  $\ddagger$  indicates values significantly lower than control,  $p < 0.05$ ).

ERK dual-phosphorylation in the presence of heparin is also shown in Fig. 3 (closed squares). Relative to serum-stimulated control, ERK activation is significantly inhibited in the presence of heparin. ERK phosphorylation was rescued in a dose-dependent fashion by supplementation of FGF-2, indicating that the relative concentrations of FGF and heparin directly influence the activation state of downstream signaling molecules, such as ERK. Moreover, in the presence of heparin, 10 ng/ml of FGF-2 stimulated ERK dual-phosphorylation by  $\sim 1.4$ -fold. These results indicate that, in monolayer tissue culture, high dose heparin inhibits serum-dependent activation of MAPK but allows stimulation of MAPK activity by exogenous FGF-2.

### 3.3. FGF-2 inhibits markers of myofibroblast activation

Given that FGF-2 rescues the heparin-mediated inhibition of ERK activity (Fig. 3) and also the reports that FGF-2 can diminish the myofibroblast phenotype in fibroblasts from many tissues [4,27–29], we were interested in the effect of FGF-2 treatment on VICs. Fig. 4 shows the effect of FGF-2 (0–100 ng/ml) on myofibroblast activation, as measured by  $\alpha$ SMA expression, in the absence or presence of excess soluble heparin (100  $\mu$ g/ml). FGF-2 suppresses  $\alpha$ SMA expression in control cultures (100 ng/ml, open squares,  $p < 0.05$ ). Additionally, FGF-2 inhibits heparin-mediated myofibroblast activation in a dose-dependent fashion (closed squares). Based on the MAPK activation results presented in Fig. 3 for these same heparin/FGF-2 doses, these data suggest that, in VICs, ERK phosphorylation is inversely proportional to myofibroblast activation.

While  $\alpha$ SMA expression is a classical myofibroblast identifier, increased levels of ECM production that accompanies activation are a useful bio-marker of functional

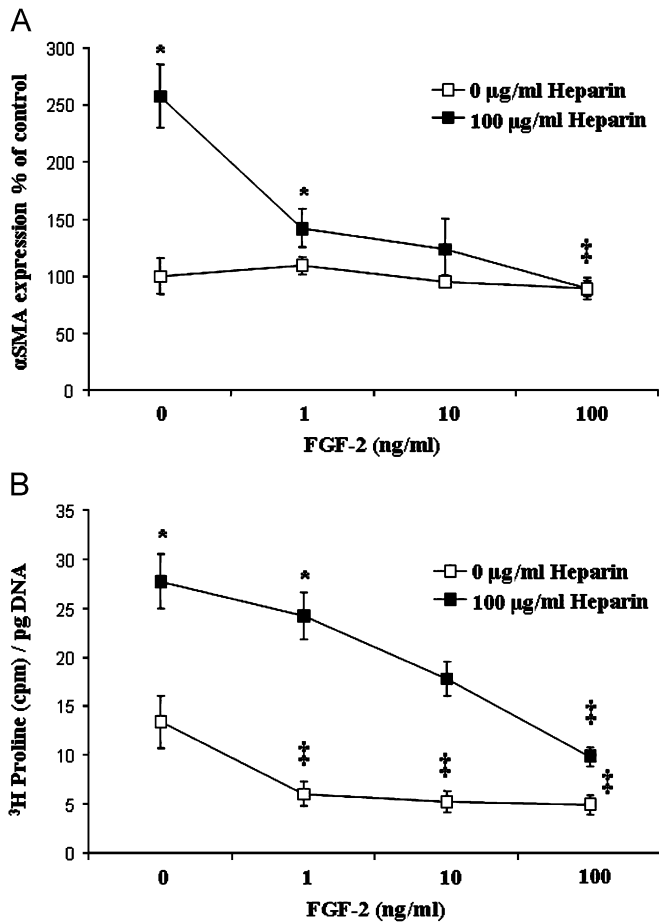


Fig. 4. (A) Valvular interstitial cell (VIC)  $\alpha$ SMA expression as a function of FGF-2 concentration (0–100 ng/ml) in the presence (■) and absence (□) of soluble heparin (100  $\mu$ g/ml). (B) Collagen matrix production, as measured by tritiated proline incorporation into extracellular matrix, as a function of FGF-2 concentration (0–100 ng/ml) in the presence (■) and absence (□) of soluble heparin (100  $\mu$ g/ml). (\*Indicates values significantly higher than control, <sup>‡</sup>indicates values significantly lower than control,  $p < 0.05$ ).

myofibroblast activity. In fibrotic diseases associated with myofibroblast overactivity, there is excess accumulation of collagenous ECM. As such, we chose to monitor VIC collagen production via radiolabeled proline incorporation into a biosynthesized matrix. It is established that TGF- $\beta$ 1 treatment of VICs increases ECM synthesis [30], but the effects of high-dose heparin treatment have not been investigated. We found that heparin-treated VICs produce significantly more collagen than untreated controls, as shown in Fig. 4B (closed squares). FGF-2 significantly inhibited this collagen overproduction in a dose-dependent manner, and significantly reduced proline incorporation below levels of untreated controls at the highest concentration tested (100 ng/ml FGF-2).

#### 3.4. Cell-surface HSPGs are implicated in FGF-2 function

The sulfation of cell-surface HSPGs is critical for their interaction with ligands such as FGF-2 [9]. To investigate

the role of HSPGs in VIC responses to FGF-2, cells were treated with chlorate, which inhibits the sulfation of heparan sulfate chains. As illustrated in Fig. 5, chlorate treatment leads to the upregulation of  $\alpha$ SMA in VICs, and FGF-2 is unable to mitigate this upregulation, indicating that cell-surface HSPGs are implicated in FGF-2 function and also that some basal activity is mediated through HSPGs to suppress  $\alpha$ SMA expression.

#### 3.5. Heparin-functionalized gels induce myofibroblast activation

Differential regulation of the MAPK pathway by FGF-2 and heparin (dose greater than 10  $\mu$ g/ml) appears to control the myofibroblast properties of VICs (Figs. 2–4). To exploit this mechanism, bioactive material surfaces were developed through incorporation and immobilization of heparin in PEG hydrogels. Because unmodified PEG gels [20] and heparin-modified gels were found to be non-adhesive for VICs, the RGDS peptide was incorporated into all gel formulations to ensure uniform attachment of cells to both control (RGD-PEG) and heparin-modified (RGD-PEG-Hep) gels.

VICs adhesion was similar between RGD-PEG and RGD-PEG-Hep gels, but cell morphology differed on the two substrates (Fig. 6A and B). VICs on heparin-modified assumed a flattened and highly spread morphology with less tapered processes, whereas VICs on gels fabricated without heparin retained a spindle shape. Also seen in Fig. 6, VICs adhered to control gels express very little  $\alpha$ SMA. In striking contrast, VICs seeded on gels that included heparin display prominent  $\alpha$ SMA-positive stress fibers.

Additionally, we tested the dependence of myofibroblast differentiation on the concentration of incorporated heparin in PEG gels. For these experiments, heparin concentration was varied between 0 and 1 mmol/L in steps of 0.25 mmol/L (initial macromer concentrations). As VIC function is altered by material stiffness [31], similar material properties between conditions were maintained by holding total GAG concentration constant. This was

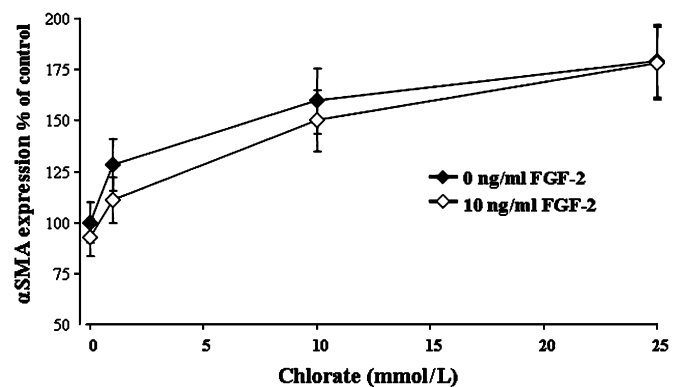


Fig. 5.  $\alpha$ SMA expression of VICs as a function of inhibition of HSPG sulfation with sodium chlorate treatment (0–25 mm) in the absence (◆) and presence (◇) of FGF-2. Chlorate-treated cells with and without FGF-2 (10 ng/ml) were not statistically different ( $p > 0.05$ ).

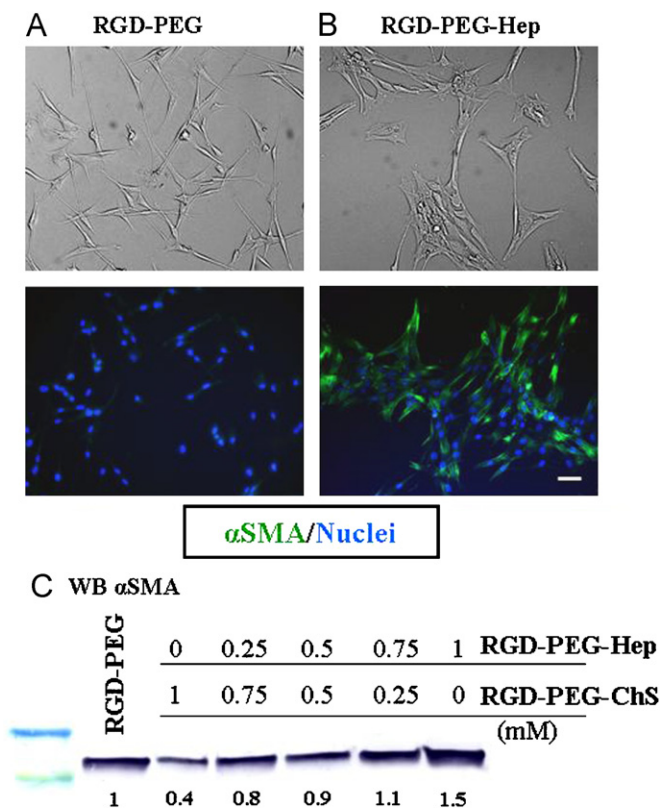


Fig. 6. Heparin was chemically modified with multiple methacrylate groups(17) and polymerized with PEG diacrylate and RGD to make heparin-modified gels (RGD-PEG-Hep). Control gels lacked heparin (RGD-PEG). (A,B) Hydrogels containing the adhesive ligand RGD (1.5 mM) (A), or RGD in combination with 1 mM heparin (B) were seeded with VICs and analyzed after 48 h. Immunofluorescent staining of  $\alpha$ SMA is shown in green, with DAPI counterstain of nuclei shown in blue (scale bar 10  $\mu$ m). (C) Western blot (WB) analysis of  $\alpha$ SMA expression as a function of hydrogel composition. All hydrogels contained the adhesive RGD ligand (1.5 mM). Chondroitin sulfate methacrylate and heparin methacrylate were covalently incorporated into hydrogels at a total summed concentration of 1 mM, ratios of ChS:Hep as follows left to right: 1:0, 0.75:0.25, 0.5:0.5, 0.25:0.75, 0:1. Semi-quantitative densitometric analysis of band intensity is presented as fraction of control  $\alpha$ SMA expression, and listed below each band.

accomplished by supplementing the gels with ChS to a total concentration of 1 mM total GAG (heparin + ChS = 1 mM). All gel formulations again contained RGDS to mediate cell adhesion. In Fig. 6C, equal protein lysates were separated by gel electrophoresis and  $\alpha$ SMA protein was detected by western blot. A trend of increasing  $\alpha$ SMA expression with increasing heparin content was observed, indicating that the effect on myofibroblast activation was specific to heparin and not a general phenomenon related to incorporation of charged GAGs within the hydrogel.

### 3.6. Heparin-functionalized gels inhibit MAPK activation

Heparin-modified hydrogels were found to actively influence VIC myofibroblast activation (Fig. 6). Studies performed in monolayer culture demonstrated that soluble heparin could inhibit serum-dependent MAPK signal

transduction (Fig. 3). In this section, corresponding studies were performed to examine the status of MAPK signaling when VICs were cultured on heparin-modified PEG gels. As seen in Fig. 7A, cells cultured on control gels, in serum-containing media, stain strongly for phosphorylated, active ERK.

In contrast, very little staining was obtained when cells were cultured on heparin-functionalized gels (7B). These results suggest that heparin inhibits serum-dependent activation of MAPK both when supplied as a soluble factor and also when immobilized within a PEG gel.

Additionally, FGF-2 treatment was utilized to assess the ability of the adherent cells to respond to soluble signals. The results, presented in Fig. 7C, confirm that cells cultured on heparin gels upregulate the myofibroblast marker  $\alpha$ SMA. The inclusion of FGF-2 (100 ng/ml) in the culture medium was sufficient to suppress  $\alpha$ SMA expression both on control surfaces and on heparin-modified surfaces; however, the fold reduction in  $\alpha$ SMA expression upon FGF-2 stimulation was greater for the heparin-modified gels than controls (8.5-fold and 2.5-fold, respectively). This enhanced response indicates a possible synergy between FGF-2 and heparin in this gel composition.

As heparin is known to bind to many proteins, among them FGF-2 and TGF- $\beta$  [32], additional studies were performed to investigate the role of serum proteins in the observed activation of VICs to myofibroblasts on heparin-modified gels. To this end, VICs were cultured on control and heparin-modified gels in serum-free media and in 15% serum-containing media. As shown in Fig. 7D, we found heparin-modified gels to induce myofibroblast activation in both serum conditions; however, serum-starved cultures upregulated  $\alpha$ SMA by a factor of  $\approx 2$ , while serum-stimulated cultures upregulated  $\alpha$ SMA by a factor of  $\approx 3$ . Additionally, serum enhanced the expression of  $\alpha$ SMA on control RGD-PEG gels; indicating that serum generally has a myoactivating effect. The activating effects of serum, however, were enhanced on the heparin-modified gels (1.6 fold increase in  $\alpha$ SMA on RGD-PEG, 2.4-fold increase on RGD-PEG-Hep). These results suggest that interaction of serum factors with the heparin gel contribute to the formation of the myofibroblast phenotype, that the heparin gel enhances the effects of serum, but also that heparin-modified gels promote myofibroblast activation in the absence of serum factors. Given the presence of TGF- $\beta$ 1 in serum (2 ng/ml, determined by lot-specific ELISA), potentiation of  $\alpha$ SMA expression in the presence of serum may be due to additional TGF- $\beta$ 1 signaling, or activation of latent TGF- $\beta$  to its active form through interactions with heparin [33].

## 4. Discussion

Initially, biomaterials were created based on their potential to be inert cell carriers supporting 3D cell culture with minimal biological activity. In contrast, recent trends in regenerative medicine and cell culture seek to actively

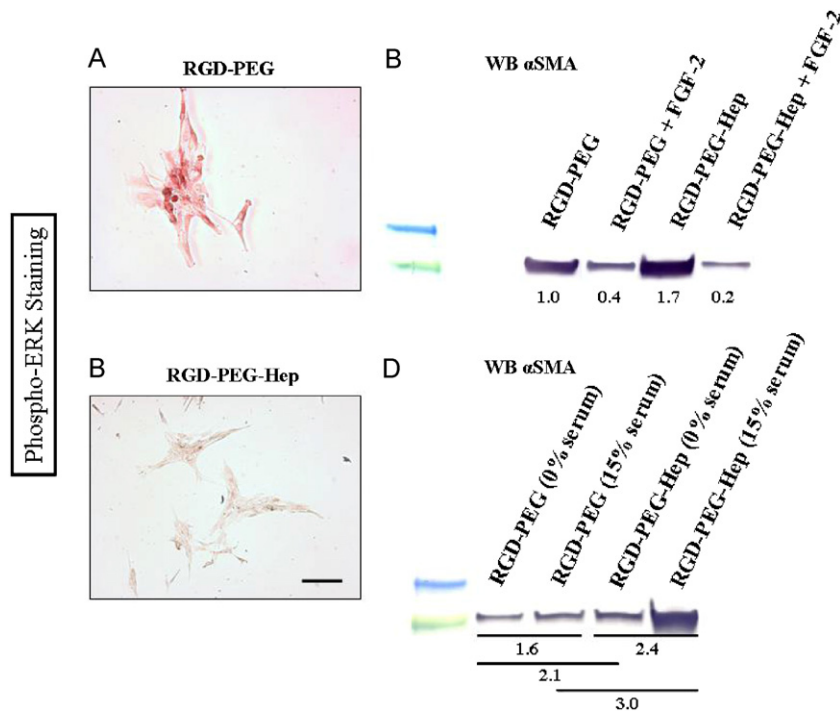


Fig. 7. (A, B) Hydrogels containing the adhesive ligand RGD (1.5 mM) (A), or RGD in combination with 1 mM heparin (B) were seeded with VICs. Adherent cells were stimulated with FBS before immunocytochemical staining of phosphorylated ERK1 and ERK2 (red) (scale bar 10 μm). (C) Western blot (WB) analysis of VIC  $\alpha$ SMA expression as a function of hydrogel composition and FGF-2 delivery (100 ng/ml). Hydrogel compositions same as in A and B. (D) WB analysis of VIC  $\alpha$ SMA expression as a function of hydrogel composition and serum conditions. VICs were seeded on hydrogels in 0% FBS (serum-starved) and 15% FBS (serum-stimulated). Semi-quantitative densitometric analysis of band intensity is presented as fraction of RGD control, and listed below each band.

regulate cellular functions by recapitulating, *in vitro*, a selected critical environmental factor present *in vivo* during development or remodeling [34]. Common themes of this approach are delivery of growth factors and soluble effectors, as well as presentation of insoluble integrin ligands to engage cell-surface adhesion receptors. New developments in biomaterial design have focused on creation of substrates with temporal and spatial complexity, such as stimulus-sensitive materials that interact with cell-produced factors or materials that contain cryptic signals exposed after defined stimuli. While elegant, these strategies require significant pre-engineering. Thus, even with precise control over the extracellular microenvironment, creating materials that manipulate intracellular cellular signaling events may circumvent many challenges posed to biomimetic strategies. In the present study, a novel strategy to regulate cell signaling through material/growth factor receptor interactions was explored. To our knowledge, this is the first study attempting to regulate intracellular signaling through growth factor-independent manipulation of growth factor receptors at a cell/material interface.

Basic FGF signaling appears to be a critical signal for negative regulation of the VIC myofibroblast phenotype [6,7] as well as a modulator of wound repair processes [35]. Due to the functional similarities between heparin and HSPG co-receptors, which bind both FGF-2 and FGFRs, exogenous heparin was explored in this study for its

potential to regulate FGF-2-dependent intracellular signaling. In monolayer tissue culture studies, soluble heparin was found to inhibit serum-dependent ERK phosphorylation and resulted in myofibroblast activation in the form of increased  $\alpha$ SMA stress fiber formation and matrix production. Further, these myofibroblast markers were suppressed when FGF-2 was delivered to stimulate MAPK activity. Chlorate treatment, which disrupts the sulfation of endogenous HSPGs, also significantly increased  $\alpha$ SMA expression indicating that cell-surface HSPGs and their ligands are important for basal repression of the myofibroblast phenotype. Translating this knowledge of the importance of heparan sulfates in repressing myofibroblast activation, heparin-functionalized gel surfaces, rendered cell-adhesive by the RGD integrin ligand, were created to modulate VIC activation. Immobilization of heparin on and within gels led to spontaneous activation of adherent VICs to an  $\alpha$ SMA-positive myofibroblast phenotype. Further, the relative amount of  $\alpha$ SMA expression was manipulated by varying the concentration of heparin within the PEG hydrogel. Serum-dependent MAPK activation was assessed by anti-active-ERK immunocytochemistry. Cells adhered to control RGD-PEG hydrogels were reactive for active MAPK; however, cells on RGD-PEG-heparin gels were relatively inhibited and showed little reactivity to the phospho-specific antibody. We infer that inhibition of MAPK on heparin-modified PEG gels is correlated with the myofibroblast phenotype,

as the upregulation of  $\alpha$ SMA protein expression on heparin-modified hydrogels was inhibited through delivery of the MAPK activating growth factor FGF-2, but maintained in serum-free conditions.

While heparin-modified PEG gels induced  $\alpha$ SMA upregulation in the absence of serum, the use of serum-containing media was found to potentiate the gel bioactivity. These results suggest that the efficacy of heparin-mediated phenotype modulation is dependent on the mixture of extracellular growth factors and cytokines. In fact, there are a growing number of studies utilizing heparinized gels for growth factor sequestration or delivery [36]. It is likely that both cell-secreted proteins and serum components, such as TGF- $\beta$ , are interacting with the heparin. On the other hand, lack of serum may itself affect MAPK activation. Serum starvation has been associated with reduced MAPK activation in cardiac fibroblasts, but also with increased phosphorylation of the stress-responsive MAPKs JNK and p38, and caspase-7 activity [37]. Thus, the relatively diminished differentiation seen here during serum-starvation on heparin-functionalized gels may be a result of initiation of stress-responses as opposed to inhibition of mitogenic stimuli. Future work will be directed to the identification of serum factors that are modulated by heparin, as well as to the study of MAPK signaling during serum starvation. As stated previously, likely targets in serum include TGF- $\beta$  and fibronectin, two factors known to bind to heparin and positively regulate myofibroblast activation.

The bioavailability of FGFs is a function of the number and location of binding heparin/heparan sulfate GAGs in the pericellular and extracellular environment. Exogenous heparin has many effects on FGF-2 physiology due to its structural similarity to heparan sulfate, and the possibility exists to manipulate the activation of FGF receptors by controlling the concentration, availability, and chemistry of exogenous heparin GAGs. As presented in Fig. 1, two strategies were employed in this study to use heparin as an external regulator of FGF-2 signaling. In the first, soluble heparin was delivered at high concentration to compete with FGFRs and HSPGs for binding of FGF-2. In the second strategy, gel-immobilized heparin was presented in the context of an RGD-adhesive substrate. In both cases, heparin was found to inhibit the patency of signaling through MAPK/ERK, implying that heparin was interfering with FGF signaling; however, other heparin-binding growth factors that activate MAPK may have been equally affected. Immobilized heparin may be interacting with cell-surface FGFRs at the cell/gel interface, creating a novel link between material and growth factor receptor. Because heparin is covalently tethered into the gel, this interaction may inhibit receptor internalization or reduce the mobility of the receptor within the plasma membrane. As clustered multimers of FGF-2/FGFR/HSPG signaling complexes represent a mature signaling complex [38], changes in FGFR membrane diffusivity may have profound effects on overall signaling activity.

## 5. Conclusions

Although there is great interest in creating functional heart valve tissue equivalents as a clinical therapy, the challenge of controlling myofibroblast activation within synthetic culture environments remains. In the present study, heparin-functionalized PEG gels were created that lowered the threshold for VIC myofibroblast activation through inhibition of FGF-2-dependent deactivation signaling through MAPK. In future studies, these gels will be explored as a culture platform for temporal regulation of myofibroblast activation, potentially allowing on/off control of the myofibroblast phenotype as will be required if these cells are to be utilized in tissue engineering applications. Additionally, selective degradation of these gels by heparinase could allow tailored delivery of soluble heparin fragments and temporal control over the gel's heparin content, which was demonstrated herein to influence the relative proportions of  $\alpha$ SMA-expressing myofibroblasts. This work represents an exciting new direction in biomaterial design focused on material-based regulation of growth factor receptor activity.

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